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Lignocellulose-Degrading Enzymes from Solid State Fermentation of Straw with Pleurotus Fungi and Their Industrial Applications

Conducted by Krystyna Sosulski

Funded by the Agriculture Development Fund

Saskatchewan
Research Council

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EXECUTIVE SUMMARY

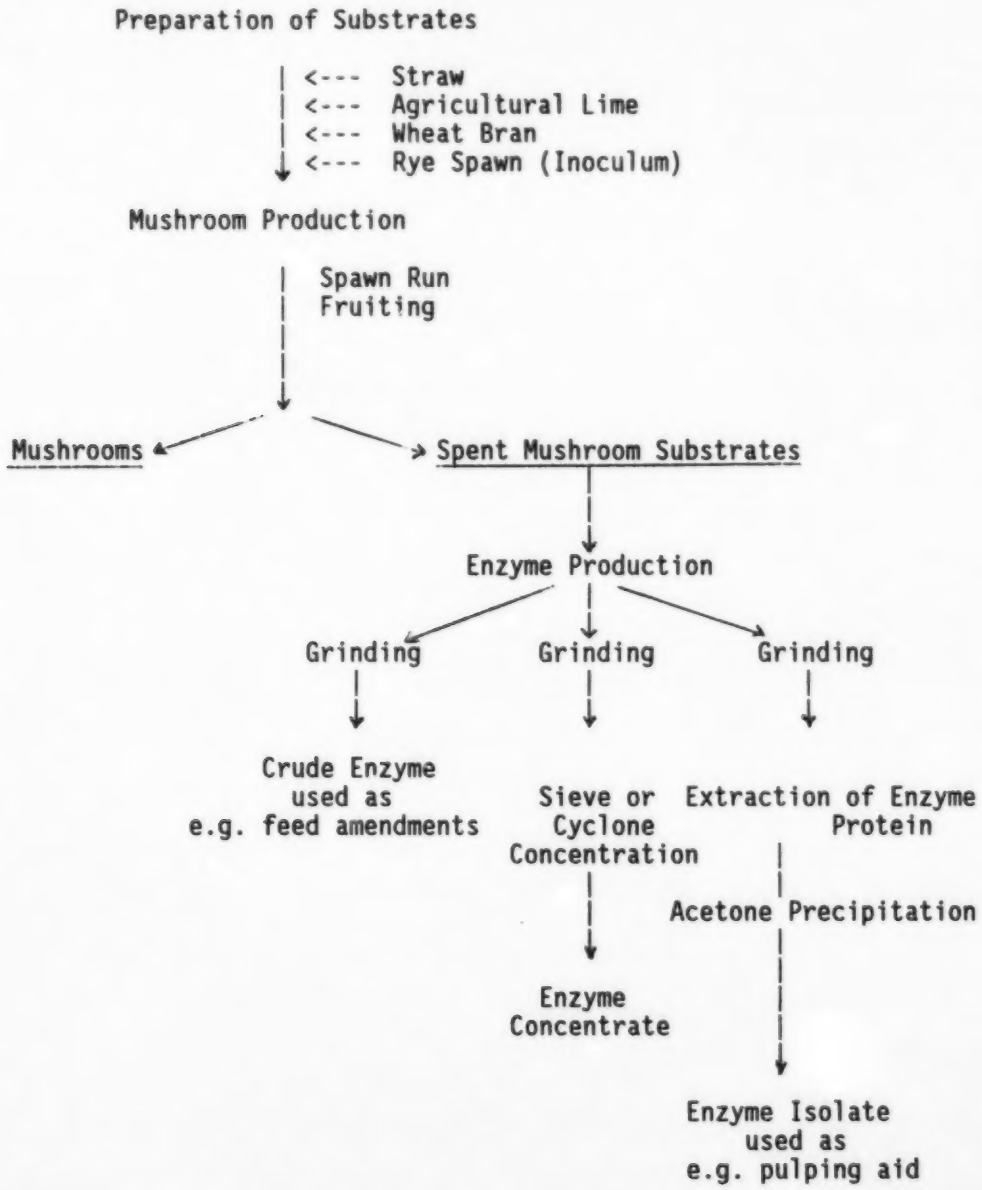
This study was undertaken as the continuation of the project, funded by the Agriculture Development Fund, on "Experimental Production of Pleurotus (oyster) mushrooms in potash mines of Saskatchewan" and in anticipation of the development of a mushroom industry in the province. A by-product of the mushroom industry, the spent substrate in which the mushrooms grow, accounts for up to 50% of the original raw material and is a waste difficult to dispose of. This product, however, contains hydrolytic enzymes, released into the substrate by fungi, and these enzymes could be recovered for various industrial applications.

Enzymes are currently produced mainly by a submerged culture in a fermentor or by a solid-state fermentation process. Both methods are expensive and require capital intensive equipment and/or environmentally-controlled space.

A new approach to enzyme production is to recover enzymes from spent, straw-based substrates after harvest of mushrooms (Figure 1). The cost of production of enzymes would be much less than using solid state or submerged fermentation processes since the mushroom sales would pay for all costs except for enzyme isolation. Low production cost could improve the economics of enzyme use in industry.

Spent substrates consisting of fermented wheat straw and supplements, were evaluated for content and composition of enzymes, after production of oyster (Pleurotus sajor-caju and P. ostreatus florida) mushrooms. The best conditions for extraction of hemicellulase enzymes were: ratio of substrate to water 1:15, pH of extraction 7.0, temperature and time of extraction 20°C and 1 hr.,respectively. Extracts from mushroom substrates were evaluated for the following enzyme activities:

Figure 1. Outline of Processes for Production of Mushrooms and Enzymes



a) hemicellulases (endo-xylanase, endo-mannanase, endo-arabinosidase, β-xylosidase),

b) cellulases (exo-cellulase, endo-cellulase, β-glucosidase),

c) enzymes hydrolysing water-soluble polysaccharides (α-arabinosidase, α-galactosidase, β-galactosidase, α-mannosidase, β-mannosidase and pectinase),

d) enzymes participating in lignin hydrolysis (laccase, polyphenoloxidase and peroxidase).

Spent substrates of the mushrooms tested were found to be a rich source of hemicellulase enzymes. P. ostreatus var. florida contained more endo-xylanase than P. sajor-caju. Enzyme activities in spent mushroom substrates could be "custom" tailored by modification of substrate composition:

a) protein and hemicellulose-enriched substrates contained more of endo-xylanase and less of exo- and endo-cellulases than control,

b) increased starch content in substrate promoted production of cellulase enzymes.

Conditions of enzyme recovery also influenced enzyme activities in the extract, e.g. extraction at pH 7.0 increased the activity of endo-xylanase while pH 5.6 used for extraction promoted recovery of exo-cellulase.

Enzymes extracted from spent mushroom substrate contained greater activities of hemicellulases (endo-xylanase and endo-mannanase) and β-glucanase, per protein unit, and were superior to commercial hemicellulases. They also contained low cellulase activities.

High hemicellulase and low cellulase activities in the enzymes produced indicated potential for their use in the pulp and paper industries, for

biopulping and biobleaching of kraft pulp. In both processes hemicellulases would cleave lignin-hemicellulose linkages, releasing lignin and hemicellulose from cellulose pulp. Researchers at PAPRICAN (Pulp and Paper Research Institute of Canada) identified several fungi as sources of enzymes for the biological production of pulp. However, these enzymes need to be produced by submerged or solid state fermentations and would be too expensive to commercialize. The enzymes recovered from spent substrate would be much cheaper than other enzymes identified for biopulping or biobleaching. It should be noted here that biological pulping and bleaching, using any source of enzymes, are not in commercial practice yet, but both are being actively pursued by the industry's research institute.

Crude substrate-derived enzymes could also be used as a digestion aid for ruminants fed a low quality roughage. Laboratory experiments indicated that total hydrolysis of fiber with enzymes can be increased from 53 to 84%, by pre-hydrolysis of fiber with hemicellulases (Sosulski, unpublished results). These hemicellulases hydrolyse hemicellulose and expose cellulose fiber to enzymes or rumen microorganisms.

ABSTRACT

Spent substrates, after production of oyster (Pleurotus sajor-caju and P. ostreatus florida) mushrooms, were evaluated for content and composition of enzymes remaining in the substrate.

The conditions of enzyme extraction were as follows: ratio of substrate to water was 1:15, pH of the slurry was adjusted to 7.0 and extraction at 20°C was carried out for 1 hr.

The following enzymes were evaluated: hemicellulases (endo-1,4-ß-D xylanase, endo-1,4-ß-D mannanase and ß-D xylosidase), cellulases (exo-1,4-ß-D glucanase, endo-1,4-ß-D glucanase and ß-D glucosidase), enzymes hydrolysing water-soluble polysaccharides (α-L arabinosidase, α-D galactosidase, ß-D galactosidase, α-D mannosidase, ß-D mannosidase and polygalacturonase-pectinase) and enzymes participating in hydrolysis of lignin (laccase, polyphenol oxidase and peroxidase).

Spent substrates of P. mushrooms were found to be rich sources of hemicellulase enzymes. P. ostreatus florida contained more endo-1,4-ß-D xylanase than P. sajor-caju.

Enzyme activities in spent mushroom substrates compared favourably with the commercial hemicellulase and cellulase enzymes, per unit protein. Isolated enzymes from P. mushroom substrates contained higher activities of endo-1,4-ß-D xylanase, endo-1,4-ß-D mannanase, ß-D xylosidase (hemicellulases) and lower activity of exo-1,4-ß-D glucanase (cellulase) than commercial enzymes.

An independent study conducted at the SRC indicated that crude enzymes of P. fungi could be used as a digestion aid for ruminants fed a low quality roughage. Hydrolysis of feed hemicellulose by the P. hemicellulases would

expose the cellulose fraction of fiber and make it more accessible to the microorganisms in the rumen.

Isolated P. enzymes were high in hemicellulase and low in cellulase activities, which is one of the requirements for enzymes used in a pulp and paper industry for biological pulping and bleaching. Enzymes recovered from spent mushroom substrate, a by-product from mushroom production, could be an attractive and cheap alternative to enzymes which are specially produced for biopulping. Based on the results presented in this report, discussions are to be undertaken with the staff of PAPRICAN regarding further evaluation and study of potential for these enzymes in the pulp and paper industry.

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INTRODUCTION

Hydrolytic enzymes occur in all living systems where they catalyse degradation of macromolecules such as starch, protein, fiber and lignin into nutrients which provide energy and substrate for cell renewal. Microorganisms, among them bacteria, yeast and fungi, release hydrolytic enzymes into the substrate from which they can be recovered directly for commercial use.

Over the past two decades considerable research has been focused on extracellular enzymes produced by a variety of microorganisms and especially on methods to increase enzyme yield and activity by genetic and environmental manipulations. Enzymes have recently become an important part of a number of industrial and consumer processes and they appear to offer great potential in many other controlled, pollution free applications.

Production of enzymes in the USA has increased at an annual rate of 7.7% and will reach a value of $450 million by 1992 (Chem. Market Report, 1989). Of the hundreds of known enzymes, however, only sixteen are of major commercial importance. About 40% of the total trade is in amylases, glucose isomerases and amyloglucosidases used in starch hydrolysis and conversion, 30% is in proteolytic enzymes used in the detergent industry and the remaining 30% includes pectinases and cellulases (Layman, 1983). The market share of proteases is expected to grow substantially, but cellulases will also have a greater portion of the market as the processes for hydrolysis of waste cellulose to glucose are becoming commercially important.

Studies of the cellulose degrading fungi Trichoderma and Aspergillus spp., led to commercial production of cellulases. The majority of cellulases currently available on the market are produced by Aspergillus niger.

The same microorganisms also produce hemicellulases, ß-glucanases and pectinases. These commercial enzymes, however, failed to hydrolyse hemicellulose in straw, alfalfa and slender wheatgrass (Sosulski et al., 1984). They lack the ability to hydrolyse lignin which, in turn, limits hydrolysis of fiber and makes biodegradation of many plant material with these enzymes quite inefficient.

Studies on microbial delignification of biomass identified white-rot fungi as organisms which are capable of preferentially degrading the lignin portion of fiber (Ander and Eriksson, 1977; Eriksson, 1978; Kirk and Kelman, 1965; Leisola et al., 1985; Pilon et al., 1982; Rouau and Odier, 1986; Yang et al., 1980). Most research, however, has focused on enzymes from Phanerochaete chrysosporum, a non-commercial edible mushroom. Enzymes of this and other fungi could be recovered from the substrate after colonization by mycelium or produced in a submerged culture in a fermentor. Both methods of enzyme production are expensive and would require environmentally-controlled space or capital-intensive equipment.

Pleurotus fungi are known for their ability to utilize all constituents of wood and other fibrous plant material. Studies conducted at the SRC Biomass Resources Laboratory indicated hydrolysis of almost 90% of hemicellulose, 50-60% of cellulose and 60-70% of lignin in straw during four weeks of mushroom production (Sosulski et al., 1985). A portion of the enzymes released by mycelium onto the substrate would still be active at the end of fruiting and could be recovered after mushroom harvest. The number of flushes of mushrooms harvested before recovery of enzymes would depend on yield and value of mushrooms and the quality, type and value of enzymes. The cost of production of enzymes would be much less, after

mushroom harvest, than by using solid state or submerged fermentations. A low cost production system would improve the economics of enzyme use in certain applications. Hemicellulases from oyster mushrooms could be used by the pulp and paper industry in biopulping, production of "dissolving" pulp or in biobleaching. All of these processes require removal of hemicellulose to yield a superior quality cellulose paper.

OBJECTIVES

Therefore the objectives of this study are to identify the activities and characteristics of enzymes present in spent mushroom substrates, as influenced by species of P. mushrooms and conditions of growth, including supplementation of a wheat straw substrate with protein and level of inoculum. Also, the method(s) of production of crude, concentrated and isolated enzymes will be evaluated in terms of their potential use.

MATERIAL AND METHODS

Material

Spent mushroom substrates of Pleurotus sajor-caju (PRL 2725, ATCC 32078 and 32771) and P. ostreatus, var. florida were air dried at room temperature, ground to pass a 1 mm screen and stored at room temperature in air-tight containers (Appendix 1 and 2). These samples were collected during the project "Experimental production of Pleurotus (oyster) mushrooms in potash mines of Saskatchewan", funded by the Agriculture Development Fund (Sosulski, 1988).

Proximate Analysis

Enzyme protein was determined using the Protein Assay Kit, P-5656, from Sigma Chemical Co., after development of colour with the Lowry reagent. Protein content in enzyme extract was extrapolated from the standard curve constructed for bovine albumin.

Moisture and ash contents of spent substrates were determined by air drying at 105°C and in the muffler furnace at 550°C, resp., according to procedures outlined in AOAC (1975).

Lipids were determined by hexane extraction of spent mushroom substrates in a Soxhlet apparatus (AOAC 1975).

Fiber content and composition were determined using the Goering and Van Soest (1970) procedure for determination of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL). Hemicellulose was estimated as the difference between NDF and ADF, while cellulose was reported as the difference between ADF and ADL.

Commercial Enzymes

Enzeco Hemicellulase from Enzyme Development Co., NJ. and Hemicellulase from Miles Laboratory Inc., IN. were used to compare their activities with enzymes extracted from spent substrate of P. mushrooms. Mixed activity enzyme, SP-249, from NOVO Industri A/S, Denmark and cellulases: Biocellulase from Biocon Biochem., Ireland; Cellulase A from Amano Enzymes, Japan; Celluclast from Novo Industri A/S, Denmark and Fungal Cellulase from Hughes and Hughes, England were also included in the study.

Enzyme Extraction

To determine conditions for enzyme extraction from spent mushroom substrates, samples were slurried in water, the ratio of substrate to water being 1:15, and the pH of the slurry was adjusted with 4N HCL and 4N NaOH from 2.0 to 9.5 in 0.5 unit intervals. Extractions were carried out by stirring at room temperature for 1 hr, after which extracted enzymes were separated from the residue by centrifugation. Enzyme extracts were analyzed for total and true protein contents using the Lowry reagent (Sigma Chemical Co.).

The best conditions for extraction of enzyme protein were pH 7.0, temp. 20°C and a water to substrate ratio of 1:15.

Enzyme Activities

Enzyme activities were determined in protein extracts obtained from spent mushroom substrates. The influence of mushroom species and strains, protein supplementation of substrate and level of inoculum on enzyme activities were investigated.

Finally commercial enzymes were evaluated for their hemicellulase and cellulase activities which were compared with those of enzymes extracted from spent substrates of Pleurotus fungi.

Enzyme activities were determined according to the following procedures:

Hemicellulases

- **Endo-1,4-ß-D xylanase** activity was determined according to the procedure of Linko et al. (1977) using xylan from Larchwood (Sigma Chemical Co.). The unit activity was expressed as µM of reducing sugars (RS), measured as xylose, per min, per g of spent mushroom substrate.
- **Endo-1,4-ß-D mannanase** activity according to Linko et al. (1977) was determined using mannan from S. cerevisiae (Sigma Chemical Co.) as a substrate. One unit of activity of enzyme, extracted from 1 g of spent mushroom substrate, released 1 µM of mannose, measured as RS, during 1 min of reaction.
- **ß-D Xylosidase** activity was determined by hydrolysis of p-nitrophenyl ß-D xylopyranoside (Sigma Chemical Co.) as described by Rouau and Odier (1986). One unit of activity was expressed as µM of product released per min by enzyme extracted from 1 g of spent mushroom substrate.
- **Endo-1,4-ß-D arabinosidase** activity was determined using arabinoglactan (Sigma Chemical Co.) following the procedure of Scharma et al. (985). One unit of activity was expressed as µM of product released per min by enzyme extracted from 1 g of spent substrate.

Cellulases

- **Exo-1,4-β-D glucanase** (exo-celiobiohydrolase) activity was determined as avicellase and filter paper activity.

a) Avicellase activity was determined according to Desrochers et al. (1981) using a solution of Avicel cellulose as a substrate.

b) Filter Paper activity (FP) was determined according to the procedure of Mandels and Sternberg (1975) using Whatman filter paper as a substrate.

Reducing sugars liberated from substrates were measured with dinitrosalicylic acid solution and enzyme activities were expressed as µM of RS liberated per min by enzyme extracted from 1 g of spent mushroom substrate.

- **Endo-1,4-β-D glucanase** (endo-cellobiohydrolase) activity was determined as carboxymethylcellulase according to the procedure of Mandels and Weber (1969) using carboxymethylcellulose as substrate. One unit of activity was expressed as µM of RS liberated per min by enzyme extracted from 1 g of spent mushroom substrate.

- **β-D-glucosidase** activity was assayed as cellobiase according to the procedure of Sternberg (1976) based on the modification of Desrochers et al. (1981), using cellobiose as the substrate and measuring the amount of liberated glucose with Statzyme glucose reagent. Units of cellobiase activity were expressed as µM of glucose produced per min of reaction by enzyme extracted from 1 g of spent substrate.

Other Water Soluble Polysaccharides

- Esterase activity involved determination of **polygalacturonase - pectinase** activities using citrus pectin as a substrate and measuring the released reducing sugars using polygalacturonic acid as a standard (Mudgett et al., 1978).
- Activities toward p-nitrophenyl-β xylopyranoside, p-nitrophenyl - β glucopyranoside, p-nitrophenyl - α galactopyranoside and p-nitrophenyl-β galactopyranoside, p-nitrophenyl -α mannopyranoside, p-nitrophenyl -β mannopyranoside were measured as amount of p-nitrophenol released from PNP sugar analog per min per g of spent mushroom substrate.

Enzymes Associated with Lignin Degradation

- **polyphenol oxidase** was determined according to the procedure of Kirk and Kelman (1965) using catechol as the substrate. Activity of enzyme is reported as an increase in absorbance, at 395 nm, in units of absorbance.
- **peroxidase** activity was determined by oxidation of syringaldazine in ethanol-peroxide solution (Ander and Eriksson 1976). One enzyme unit is the amount of enzyme that oxidizes 1 μM of syringaldazine per min.
- **laccase activity** was analyzed according to Ander and Eriksson (1976) as modified by the same authors (1977). The syringaldazine was oxidized in ethanol-hydrogen peroxide-acetate buffer mixture, pH 5.0. One unit of enzyme activity was defined as the amount of enzyme that oxidizes 1 μM of syringaldazine per min.

Enzyme Preparates

Crude enzymes were obtained by grinding the whole, dried spent mushroom substrate to 1 mm particle size.

Enzyme concentrate was obtained by sieve separation of crude enzyme followed by air classification of selected (0.4-1.0 mm size) fractions into light and dense particles. The light fraction was designated as an enzyme concentrate.

Enzyme isolate was obtained by extraction of spent mushroom substrate in water, at pH 7.0 in the slurry, and at substrate to water ratio of 1:15, during 1 hr extraction at room temp. The residue was re-extracted at ratio of residue to water of 1:7, during 30 min of extraction.

Purification and concentration of isolated proteins was carried out on the Pellicon Cassette Ultrafilter from Millipore Co., fitted with a 10,000 MW cut-off membrane. Enzyme proteins were precipitated from cold solution, at 5°C, by acetone and freeze-dried.

RESULTS AND DISCUSSION

Composition of Spent Substrate

Substrates of P. sajor-caju, 3 strains, and P. ostreatus var. florida were comparable in their protein and lipid contents (Table 1). Hemicellulose content, however, was 2% higher for substrates of two of the three strains of P. sajor-caju than in P. ostreatus florida substrate. The substrate of the latter species of oyster mushroom, however, had a slightly higher cellulose and the lowest lignin contents among studied samples. Residual hemicellulose, cellulose and lignin contents indicated the substrate utilization pattern by mycelium and the potential levels of residual enzymes in spent substrates.

Enzyme Activities

The scientific name of enzymes, the EC number assigned by the Enzyme Nomenclature Committee, the basis for their assay and substrate are briefly summarized in Table 2. The following tables contain only scientific names of the enzymes; the substrates used for determination are shown only for exo-cellobiohydrolase.

Slurries of spent mushroom substrate in water were adjusted to pH levels ranging from 2.0 to 9.5 in 0.5 unit intervals, in order to determine protein solubility. It was found that acidic pH, range 2.0-4.5, resulted in a very low protein solubility. The majority of protein was extracted at pH 7.0 and a further increase in alkalinity did not increase the yield of solubilized proteins. Therefore, pH 7.0 was chosen as the optimum for protein extraction. But the native pH of the substrate in which fungi utilized the substrate constituents was 5.6, and was probably the optimum

for enzyme activity. Therefore, extracts obtained at pH 5.6 and 7.0 were evaluated for enzyme activities (Table 3).

To avoid variations in enzyme activities, due to a difference in pH of the extract, all determinations were carried out with substrates in buffer at the pH required for a particular determination.

Spent substrates of P. mushrooms contained more hemicellulose- than cellulose- degrading enzymes (Table 3). Neutral pH preferentially selected for hemicellulases, while the slightly acidic pH of 5.6 was preferred for extraction of cellulases. However, ß-D glucosidase enzyme which is required to complete hydrolyses of cellulose to simple sugars was in a greater activity at neutral than acidic pH.

Enzymes involved in degradation of water-soluble polysaccharides did not respond uniformly to the conditions of extraction (Table 3). Poly-galacturonase, α-D and ß-D mannosidases expressed lower activities in extracts made at pH 7.0 than at 5.6. However, the activities of α-D arabinosidase, α-D galactosidase and ß-D glucosidase were increased by extraction of enzymes at pH 7.0 as compared pH 5.6.

Among enzymes responsible for degradation of lignin, only peroxidase was influenced by pH of extraction process (Table 3). More peroxidase was extracted at acidic than at neutral pH.

As reported in Table 3, spent substrates of P. fungi contained considerable quantities of cell well degrading enzymes. As opposed to soft- or brown-rot fungi, which hydrolyse mainly polysaccharides, white-rot fungi P. ostreatus var. florida and P. sajor-caju also utilize lignin.

Several authors evaluated degradation of lignin by basidomycetes in a submerged culture fermentation (Leatham and Kirk 1983); Plat et al.,1984; Yang et al., 1980; Ander and Eriksson, 1975 and Leisola et al., 1985.

Cellulases were studied mainly as an example of Trichoderma viride (Linko et al., 1977; Sternberg, 1976), although other fungi were also included (Margaritis and Merchant, 1983; Hong and Namgung, 1975). Xylanases were also studied using Trichoderma viride (Linko et al., 1977) although much more effort was focused on other fungi species (Deshpande et al., 1986; Sharma et al., 1985; Biely et al., 1986; Hong 1976).

Maximum ligninase activity was determined in a 20-day old culture of P. ostreatus (Leatham and Kirk, 1983) or a 21-day old culture of P. ostreatus var. florida (Plat et at., 1984). At this time, 56% of lignin was already removed from the substrate.

Hydrolysis of cellulose is reported to require the concentrated action of at least three enzymes; exo- and endo-cellobiohydrolases and ß glucosidase (Pilon et al., 1982). Production and accumulation of cellulases is the fastest during the early stage of mycelium growth and ceases after most of the easily-available cellulose is consumed (Sternberg, 1976). ß glucosidase, however, appears much later and remains in the substrate until all fragments of cellulose, hydrolysed by cellulases, are further transformed to simple sugars.

Endo-xylanase and ß xylosidase were reported to be responsible for hydrolysis of xylan to xylo-oligasaccharides and simple sugars, resp. (Deshpande et al., 1986). However, according to Biely et al. (1986) xylan hydrolysis could not take place without a synergistic presence of esterases responsible for deesterification of acetyl-xylan. Commercially available xylan is deacetylated, therefore, esterase activity of the enzyme extract was determined using citrus pectin as substrate and is reported as pectinase (Table 3).

As reported in Table 3, P. substrates contained a considerable quantity of cell wall-degrading enzymes with hemicellulases being predominant.

The pulp and paper industry requires hemicellulases which are free of or with a minimum of cellulase activity. It is fortunate that activities in enzymes extracted from spent mushroom substrate could be "custom made" by modification of extraction pH. To maximize hemicellulase activities all further tests were conducted on enzymes extracted at pH 7.0.

Influence of Mushroom Species on Enzyme Activities

The activity of the endo-1,4-ß-D xylanase enzyme was influenced by species and strains of P. mushrooms (Table 4). Two of three P. sajor-caju strains had low endo-xylanase activity compared to the third strain, ATCC 32771, and especially to P. ostreatus var. florida.

Endo-1,4-ß-D mannanase activity was only 15-42% of that of endo-xylanase and was the highest in P. sajor-caju, ATCC 32078 (Table 4).

ß-D xylosidase activity was relatively high only in the extract from the substrate of P. sajor-caju, PRL 2725, while endo-1,4-ß-D arabanase was low for all substrates (Table 4).

Cellulose-degrading enzymes were low as compared to hemicellulases for all investigated strains and species of P. fungi (Table 4). Especially low value was for exo-1,4-ß-D glucanase (exo-cellohydrolase) activity which participated in hydrolysis of polymorphic cellulose. Lack of this enzyme suppressed activity of endo-1,4-ß-D glucanase (endo-cellobiohydrolase) in an actual lignified substrate. To determine the full potential of endo-cellohydrolase, activity was evaluated using a soluble cellulose. ß-D glucosidase was slightly higher, in all samples of P. mushroom, than exo-

cellulase determined with filter paper substrate. Most of the commercial cellulases, produced by Trichoderma viride, do not have or have only marginal activity of β-D glucosidase (Novo Handbook, 1986).

Activities of enzymes degrading water-soluble polysaccharides were similar among most P. mushrooms (Table 4). An exceptionally high value of α-D galactosidase activity was determined in P. ostreatus variety florida while a very low β-D glucosidase activity was present in P. sajor-caju, ATCC 32771.

Laccase and peroxidase activities were the highest in the extract from substrate of P. ostreatus var florida while peroxidase activity was higher in P. sajor-caju, PRL 2725 (Table 4).

Influence of Protein Supplementation on Enzyme Activities

Supplementation of the wheat straw substrate for production of oyster mushrooms with wheat bran, as a protein source, modified enzyme activities in P. ostreatus florida (Table 5). Its endo-1,4-β-D xylanase activity was increased by 23%, while endo-1,4-β-D mannanase and β-D xylosidase activities were not improved. This moderate change in hemicellulase activities, however, was followed by a 67% reduction in exo-1,4-β-D glucanase activity and a 52% reduction in endo-1,4-β-D glucanase and β-D glucosidase activities.

Supplementation of substrates with protein did not influence esterase activity, reported as polygalacturonase (Table 5). Two other polysaccharidases, α-L arabinosidase and α-D galactosidase, showed increased activities while activities of other enzymes decreased.

Activity of laccase remained unchanged, while polyphenol oxidase and peroxidase were less active in protein-supplemented substrate (Table 5).

The negative influence of protein supplementation on activities of ligninase enzymes was reported by Reid (1979), Leatham and Kirk (1983). However, no information is available on how protein supplementation of substrate changes the activities of hemicellulase and cellulase enzymes.

Wheat bran used in this study contained only 18% protein. Each gram of protein added to mushroom substrate increased, therefore, the concentration of other constituents, among them hemicellulose and cellulose, by 1.5 and 1.0 g, resp. (Sosulski, 1988). Therefore, the change in enzyme activities determined in spent substrate has to be attributed to the combined effect of protein and fiber.

Influence of Inoculum Level on Enzyme Activities

Increased level of rye grain spawn (mushroom inoculum), with an associated increase in starch content in the mushroom substrate, decreased endo-1,4-ß-D xylanase activity by a moderate 16% and endo-1,4-ß-D mannanase by 26% (Table 6). Cellulase activities, however, were slightly increased by a higher inoculum level.

Increased inoculum level had detrimental effects on enzymes degrading water-soluble polysaccharides (Table 6). However, there was a substantial increase in the activity of laccase in a substrate with the greatest inoculum level of 3.2%. Polyphenol oxidase activity also increased but to a lesser degree, while peroxidase activity was decreased.

According to Ander and Eriksson (1975) production of laccase is repressed by the presence of the easily metabolized carbohydrates, e.g.

starch. Our experiment suggests that mycelium undertakes the production of laccase immediately after exhausting the supply of available carbohydrates, which happened at the latest date for the substrate with the highest inoculum, 3.2% (Table 6).

Influence of Growth Conditions on Enzyme Activities

Production of mushrooms in the potash mine, in a micro-environment of a corridor 1000 m underground, had a substantial influence on the activities of major enzymes recovered from spent mushroom substrates (Table 7). Endo-1,4-β-D xylanase, exo- and endo- cellobiohydrolases were decreased in their activities by over 50%. Other enzymes were less, or not, affected by conditions during substrate colonization and mushroom production.

The specific environment of the mine, a combination of pressure, temperature and salt in air, modified the process of mushroom production as well. Substrates were colonized 2-3 days faster than in the growthroom and the yield of the first flush of mushrooms was improved by 39% (Sosulski, 1988).

Such a sudden production of mushrooms required an increased supply of nutrients which were probably derived from hemicellulose and cellulose rather than lignin. According to Eriksson (1977) however, cellulose and lignin are simultaneously utilized by mycelium. Other authors suggested that white rot fungi preferentially utilize lignin (Kirk and Moore, 1972). Some reports, however, indicated that utilization of lignin depends on and is improved by the supply of oxygen (Yang et al., 1980) and is decreased by supplementing of substrate with protein sources (Reid et al., 1985; Young et al., 1980). The production of enzymes and utilization of substrate

components by mycelium and mushrooms depends also on the presence of products of hydrolysis. Monosaccharides cause catabolite repression of endo-1,4-ß-D glucanases (Eriksson, 1977). Xylose and arabinose are tolerated at greater concentrations than glucose and mannose. Simple carbohydrates were also reported to suppress production of laccase and ß-glucosidase (Ander and Eriksson, 1975). It was also suggested that phenol oxidases may function in regulating the production of both lignin and polysaccharide-degrading enzymes (Eriksson, 1977). However, some of the aromatic compounds, e.g., phenolic acids, especially at higher concentration, suppressed production of xylanase (Sharma et al., 1985). It appears that, to be able to run bioconversion process successfully and to produce enzymes or mushrooms it is necessary to learn more about the mechanisms involved in regulation of extracellular enzyme production. To successfully recover the most valuable enzymes from spent mushroom substrate one would need to evaluate their levels during various stages of mycelium growth and after one, two or more flushes of mushrooms. Also, information on the influence of medium composition and pH requirements for production of particular enzymes is essential.

Substrates evaluated in this study were exhaustively fruited to evaluate their full mushroom-growing potential in the mine or in the growthroom. However, the maximum mushroom yield per day of production, counting from the date of substrate inoculation, is reached after two flushes (Sosulski et al., 1985). Prolonged production decreases mushroom yield per day, and, as indicated here, would also deplete valuable enzymes in the substrate.

Pleurotus v.s. Commercial Enzymes

Crude extracts of P. mushroom substrates were low in enzyme activities and compared unfavourable per gram product with more concentrated commercial enzymes (Table 8). Variation in protein content were correlated with enzyme activities. Therefore, it appeared necessary to compare P. enzymes and commercial enzymes activities on the same protein content basis.

When enzyme activities were expressed per unit protein, the P. fungi were better or comparable to commercial enzymes in their hemicellulase activities (Table 9). Endo-1,4-ß-D xylanase of P. ostreatus florida was almost twice as high as that of Enzeco Hemicellulase and three times as high as Hemicellulase from Miles Lab. Inc. However, two of cellulases, Cellulase A from Amano Inc. and Fungal Cellulase from Biocon Biochem. were slightly better in their xylanase activities than the mushroom sources.

Pleurotus enzymes in addition to high endo-xylanase activity, contained high endo-mannanase and ß-xylosidase, low exo- cellobiohydrolase and high ß-glucosidase activities. Commercial enzymes did not contain even a trace of ß glucosidase.

Crude, Concentrated and Isolated Enzymes

Crude enzymes were obtained by simple grinding of the spent mushroom substrate on a Wiley mill fitted with a 1 mm screen. Particles of crude enzyme varied from 0.4 to 2.4 mm in size. The smallest particles, 0.4-0.5 mm, accounted for 60% of the total sample. Particles up to 1 mm size constituted 79% of the total sample.

Crude enzymes contained 2.3% of true protein. However, the distribution of protein in the sample depended on the size of the particles.

Small particles 0.4 mm size, contained 3.1% protein, while particles of 1.5-1.4 mm had only 2.1%. Therefore, it appears feasible to concentrate protein by sieving and further air cyclone separation into lighter and denser fractions.

Enzyme concentrates were air classified from a sieve separated small particle size fractions, 0.4-0.5 mm and 0.6-1.0 mm. Light fractions obtained by air classification accounted for 14 and 28% of the total material, resp. Protein contents were increased from 2.3 and 2.9% to 3.0 and 6.2%, resp. Xylanase activity was concentrated into the light fraction, from 5.8 to 7.8 M/min/g product and from 8.3 to 12.1 M/min/g product for P. sajor-caju and P. ostreatus florida, resp., (Table 10).

Isolation of enzyme protein was carried out at pH 7.0, in which 20% more protein was solubilized than at the native pH (pH 5.6) of spent mushroom substrate. The pH 7.0 was also more suitable to recover hemicellulases as opposed to cellulases (Table 3).

The purification process of the isolated protein on the ultrafilter, fitted with 10,000 MW membrane, was unsuccessful. Endo-xylanase activity was determined in the retentate, suggesting a very low molecular weight of enzyme extracted from P. substrate. Low molecular weight endo-xylanase was also reported by Ujiie et al. (1989). These authors indicated that losses of enzymes occurred through the same size membrane as used in this study. Therefore, isolated proteins were precipitated by acetone, separated by centrifugation and freeze-dried.

Endo-xylanase activity in the enzyme isolates was high, 37.7 and 55.7 μM/min/g of product for P. sajor-caju and P. ostreatus florida, resp. (Table 10).

The differences between the crude, concentrated and isolated enzyme preparations were not only in their activities or degrees of purity. The cost of production of crude enzymes would be negligible as compared to concentration. While the first system required only a mill, the second one involved the use of an air cyclone or sieves, in addition to the mill. The isolation process, although providing the highest activity among the enzyme preparations required capital-intense equipment.

A decision on the optimum degree of concentration and purification of enzymes has to be based on enzyme application. Crude enzymes might find a use as a digestion aid for ruminants on a poor quality roughage. Spent mushroom substrates, themselves, have an organic matter digestible in 53%, which is 29% better than straw alone.

Enzyme isolates, purified and concentrated, might be a future source of hemicellulases and cellulases in hydrolysis of lignocellulosic to simple, fermentable sugars or as a source of hemicellulases for the pulp and paper industry. A study conducted at SRC on use of P. enzymes in the hydrolysis of hemicellulose of chaff proved the superiority of P. enzymes over commercial enzymes (Sosulski, unpublished results). Yield of reducing sugars was two to three times that obtained with the commercial hemicellulases.

The results obtained in this preliminary study indicate a potential for development of hemicellulase enzymes from spent substrate of P. mushrooms. The desired, specific enzyme activities could be influenced by formulation of the substrates for production of mushrooms, e.g., protein and hemicellulose supplementation of substrate will not only improve mushroom yield but also increase xylanase activity.

Further studies are needed, based on recovery of enzymes from "fresh" spent mushroom substrate which had not been subjected to a drying process. More studies are required in the area of concentration of isolated proteins.

The submerged fermentation process for production of pure enzymes from P. fungi could be an alternative to enzyme recovery from spent substrate. Data presented in this report could provide important information regarding selection of media for mycelium growth and production of desired enzyme activities.

CONCLUSIONS

1. Spent substrates of Pleurotus fungi could serve as a source of hemicellulase enzymes. P. ostreatus florida produces greater amounts of endo-1,4-β-D xylanase than P. sajor-caju.
2. Enzyme activities in spent mushroom substrates could be "custom" tailored by modification of substrate composition.
   a) protein-hemicellulose enriched substrates contained more of endo-1,4-β-D xylanase and less of exo- and endo-1,4-β-D glucanases than control,
   b) increased starch contents in substrates promoted production of cellulase enzymes.
3. Enzyme activities in extracts from mushroom substrates depended on conditions for enzyme recovery, e.g. extraction of enzymes at pH 7.0 increased activity of endo-1,4-β-D xylanase while pH 5.6 promoted recovery of exo-1,4-β-D glucanase.
4. Isolated enzymes contained more reactive endo-1,4-β-D xylanase than crude enzyme preparates.
5. Enzymes extracted from P. mushroom spent substrate contained greater activities of endo-1,4-β-D xylanase, endo-1,4-β-D mannanase and β-D xylosidase and lower activity of exo-1,4-β-D glucanase, per protein unit, as compared to commercial hemicellulases and cellulases.
6. Crude enzymes of P. mushrooms could aid digestion of a poor quality roughage by ruminants and by hydrolysis of hemicellulose to expose the cellulose portion of fiber to the microorganisms in the rumen (Sosulski, unpublished results).

7. Isolated P. enzymes contained high hemicellulase and low cellulase activities, which indicate the potential for their use in the pulp and paper industry for biopulping and biobleaching processes. Further work is necessary to demonstrate this alternative source of enzymes for a promising new area being researched by the pulp and paper industry.

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PERSONNEL INVOLVED IN THE PROJECT

Professional:

Dr. Krystyna Sosulski	12% of total time

Technical:

Mrs. Betty Swerhone	30% of total time
Miss Ann Mair	15% of total time

STATEMENT OF EXPENDITURE

Labour Cost

Professional	$15,444.50
Technical	$11,268.86
Equipment Rental, Maintenance	$ 660.00
Material and Supplies	$ 2,453.79
Other	$ 172.85
Total	$30,000.00

Additional cost of labour required on this project was covered by the Saskatchewan Research Council. SRC contribution was $2,671.34.

Table 1. Chemical composition of spent mushroom substrates, %db.

Composition	Spent mushroom substrates			
	P. s-c PRL 2725	P. s-c ATCC 32771	P. s-c ATCC 32078	P. o. Fl ATCC 38539
Crude Protein	7.2	7.4	6.9	7.2
Lipid	2.1	2.5	2.3	2.0
Hemicellulose	7.8	7.3	5.7	5.0
Cellulose	37.4	37.8	37.9	38.8
Lignin	7.0	7.1	6.9	6.7
Ash	17.1	18.7	18.8	17.3

Table 2. Assay substrates and techniques used for determination of extracellular enzymes of Pleurotus mushrooms.

Enzymes Degrading Natural Substrates	EC No[1]	Assay Substrate	Basis for Assay
Cellulose and products of cellulose degradation			
Exo-1,4-β-D glucanase (exo-cellobiohydrolase)	3.2.1.91	Avicel cellulose	Formation of reducing sugars
Exo-1,4-β-D glucanase (exo-cellobiohydrolase)	3.2.1.91	Filter paper	Formation of reducing sugars
Endo-1,4-β-D glucanase (endo-cellobiohydrolase)	3.2.1.4	Carboxymethyl-cellulose	Formation of reducing sugars
β-D glucosidase (cellobiase)	3.2.1.21	Cellobiose	Formation of reducing sugars
Hemicellulases			
Endo-1,4- -D xylanase (endo-xylanase)	3.2.1.8	Xylan (larchwood)	Formation of reducing sugar
Endo-1,4-β-D mannase (endo-mannanase)	3.2.7.78	Mannan (S. cerevisiae)	Formation of reducing sugars
Endo-1,4- -D arabinosidase	3.2.1.55	Arabinogalactan	Formation of reducing sugars
B-D- Xylosidase	3.2.1.37	PNP-β-D xylo-pyranoside	Release of PNP
Other Water Soluble Polysaccharides			
α-L-Arabinosidase	3.2.1.55	PNP-α-Arabino-furanoside	Release of PNP
α-L-Galactosidase	3.2.1.22	PNP-α-D galac-topyranoside	Release of PNP
β-D-Galactosidase	3.2.1.23	PNP-β-D galac-topyranoside	Release of PNP
β-D-Galactosidase	3.2.1.21	PNP-β-D-gluco-pyranoside	Release of PNP
α-D-Mannosidase	3.2.1.24	PNP-α-D manno-pyranoside	Release of PNP
β-D-Mannosidase	3.2.1.25	PNP-β-D manno-pyranoside	Release of PNP
Polygalacturonase-pectinase	3.2.1(67/15)	Citrus pectyn	Formation of reducing sugars

Table 2. (continued)

Enzymes Degrading Natural Substrates	EC No[1]	Assay Substrate	Basis of Assay
<u>Enzymes Associated with Lignin Degradation</u>			
Laccase	1.10.3.2	Syringaldazine	Oxidation products
Polyphenol Oxidase	1.10.3.1	Catechol	Oxidation products
Peroxidase	1.11.1.7	Syringaldazine/ H_2O_2	Oxidation products

[1] Definitive assignment of Enzyme Nomenclature Committee.

Table 3. Enzyme activities in protein extracts made at pH 5.6 and 7.0 from spent substrate of Pleurotus sajor-caju ATCC 32078.

Enzymes (Substrates used for determination)	pH of extraction 7.0	5.6
Hemicellulase		
Endo-1,4-ß-D xylanase[1]	3.62	2.22
Endo-1,4-ß-D mannanase[1]	1.87	1.78
ß-D-Xylosidase[2]	6.00	4.00
Endo-1,4-ß-D arabinosidase[1]	0.59	0.70
Cellulases		
Exo-1,4-ß-D glucanase (filter paper)	0.84	1.86
Exo-1,4-ß-D glucanase (Avicel cellulose)	0.12	0.14
Endo-1,4-ß-D glucanase	0.90	1.87
ß-D Glucosidase	1.29	0.93
Enzymes Degrading Water Soluble Polysaccharides		
Polygalacturonase[1]	4.01	4.67
α-L-Arabinosidase[2]	15.00	11.00
α-D-Galactosidase[2]	110.00	83.00
ß-D-Galactosidase[2]	36.00	39.00
ß-D-Glucosidase[2]	118.00	75.00
α-D-Mannosidase[2]	2.00	8.00
ß-D-Mannosidase[2]	9.00	19.00
Enzymes Associated with Lignin Degradation		
Laccase[1]	0.19	0.18
Polyphenol Oxidase[3]	0.025	0.011
Peroxidase[1]	0.97	1.30

[1] µM/min/g substrate, [2]nM/min/g substrate, [3]absorbance units.

Table 4. Enzyme activities in protein extracts from spent substrates of three strains of Pleurotus sajor-caju and one strain of P. ostreatus, var. florida.

Enzymes (Substrates used for determination)	Pleurotus sajor-caju PRL 2725	Pleurotus sajor-caju ATCC 32078	Pleurotus sajor-caju ATCC 32771	Pleurotusostreatus var. florida ATCC 38539
Hemicellulases				
Endo-1,4-β-D xylanase[1]	3.81	3.45	5.81	8.28
Endo-1,4-β-D mannanase[1]	1.39	2.97	1.43	1.26
β-D-Xylosidase[2]	19.00	4.00	4.00	8.00
Endo-1,4-β-D arabinosidase[1]	0.64	0.45	0.66	0.84
Cellulases				
Exo-1,4-β-D glucanase[1] (filter paper)	0.99	1.27	1.25	2.68
Exo-1,4-β-D glucanase[1] (Avicel cellulose)	0.20	0.11	0.08	0.12
Endo-1,4-β-D glucanase[1]	2.68	2.52	2.54	4.23
β-D Glucosidase[1]	1.33	1.54	1.54	3.31
Enzymes Degrading Water Soluble Polysaccharides				
Polygalacturonase[1]	4.32	4.59	4.68	4.64
α-L-Arabinosidase[2]	9.00	7.00	12.00	10.00
α-D-Galactosidase[2]	57.00	31.00	79.00	113.00
β-D-Galactosidase[2]	22.00	15.00	35.00	36.00
β-D-Glucosidase[2]	121.00	44.00	141.00	125.00
α-D-Mannosidase[2]	15.00	4.00	9.00	16.00
β-D-Mannosidase[2]	8.00	19.00	15.00	19.00
Enzymes Associated with Lignin Degradation				
Laccase[1]	0.84	0.58	1.43	1.95
Polyphenol Oxidase[3]	0.014	0.010	0.005	0.022
Peroxidase[1]	1.56	0.71	0.97	1.10

[1] μM/min/g substrate, [2] nM/min/g substrate, [3]absorbance units.

Table 5. Enzyme activities in protein extracts from substrates of Pleurotus ostreatus var. florida (TCC 38539), supplemented with wheat bran protein at 2% level, as compared to control.

Enzymes (Substrates used for determination)	Control - 0% protein supplementation	Sample - 2% protein supplementation
Hemicellulases		
Endo-1,4-β-D xlanase[1]	8.28	10.20
Endo-1,4-β-D mannanase[1]	1.26	1.26
β-D-Xylosidase[2]	8.00	8.00
Endo-1,4-β-D arabinosidase[1]	0.84	0.66
Cellulases		
Exo-1,4-β-D glucanase[1] (filter paper)	2.68	0.89
Exo-1,4-β-D glucanase[1] (Avicel cellulose)	0.12	0.21
Endo-1,4-β-D glucanase[1]	4.23	2.04
β-D Glucosidase[1]	3.31	1.61
Enzymes DegradingWater Soluble Polysaccharides		
Polygalacturonase[1]	4.64	4.46
α-L-Arabinosidase[2]	10.00	15.00
α-D-Galactosidase[2]	113.00	126.00
β-D-Galactosidase[2]	36.00	25.00
β-D-Glucosidase[2]	125.00	67.00
α-D-Mannosidase[2]	16.00	13.00
β-D-Mannosidase[2]	19.00	16.00
Enzymes Associated with Lignin Degradation		
Laccase[1]	1.95	1.95
Polyphenol Oxidase[3]	0.022	0.011
Peroxidase[1]	1.10	0.91

Table 6. Enzyme activities determined in protein extracts from spent substrate of Pleurotus sajor-caju (ATCC 32078) inoculated with 1.6, 2.4 and 3.2% of spawn.

Enzymes (Substrates used for determination)	Inoculum Level (%) 1.6	2.4	3.2
Hemicellulases			
Endo-1,4-β-D xylanase[1]	6.38	5.34	5.33
Endo-1,4-β-D mannanase[1]	2.99	2.23	2.09
β-D-Xylosidase[2]	6.00	10.00	8.00
Endo-1,4-β-D arabinosidase[1]	0.65	0.62	0.65
Cellulases			
Exo-1,4-β-D glucanase[1] (filter paper)	1.44	1.46	1.88
Exo-1,4-β-D glucanase[1] (Avicel cellulose)	0.194	0.18	0.13
Endo-1,4-β-D glucanase[1]	2.55	2.75	2.41
β-D Glucosidase[1]	0.97	1.01	1.85
Enzymes Degrading Water Soluble Polysaccharides			
Polygalacturonase[1]	4.16	4.05	3.90
α-L-Arabinosidase[2]	8.00	23.00	22.00
α-L-Galactosidase[2]	123.00	99.00	112.00
β-D-Galactosidase[2]	61.0	48.00	62.00
β-D-Glucosidase[2]	154.00	140.00	125.00
α-D-Mannosidase[2]	10.00	15.00	14.00
β-D-Mannosidase[2]	20.00	19.00	22.00
Enzymes Associated with Lignin Degradation			
Laccase[1]	0.06	0.13	0.52
Polyphenol Oxidase[3]	0.016	0.024	0.036
Peroxidase[1]	1.43	1.10	1.05

[1] μM/min/g substrate, [2] nM/min/g substrate, [3]absorbance units.

Table 7. Enzyme activities determined in protein extracts of Pleurotus sajor-caju (ATCC 32078) grown and fruited in mine, or growthroom.

Enzymes (Substrates used for determination)	Mine	Growthroom Only
Hemicellulases		
Endo-1,4-β-D xylanase[1]	3.48	6.41
Endo-1,4-β-D mannanase[1]	4.90	4.27
β-D-Xylosidase[2]		
Endo-1,4-β-D arabinosidase[1]	0.61	0.70
Cellulases		
Exo-1,4-β-D glucanase[1] (filter paper)	0.75	1.86
Exo-1,4-β-D glucanase[1] (Avicel cellulose)	0.12	0.14
Endo-1,4-β-D glucanase	0.89	1.87
β-D Glucosidase[1]	0.89	0.93
Enzymes Degrading Water Soluble Polysaccharides		
Polygalacturonase[1]	4.50	4.67
α-L-Arabinosidase[2]	7.00	11.00
α-D-Galactosidase[2]	61.00	83.00
β-D-Galactosidase[2]	25.00	39.00
β-D-Glucosidase[2]	92.00	75.00
α-D-Mannosidase[2]	7.00	8.00
β-D-Mannosidase[2]	1.00	19.00
Enzymes Associated with Lignin Degradation		
Laccase[1]	0.65	0.00
Polyphenol Oxidase[3]	0.016	0.011
Peroxidase[1]	0.97	1.36

[1] μM/min/g substrate, [2] nM/min/g substrate, [3]absorbance unit.

Table 8. Comparison of activities of Pleurotus fungi and commercial enzymes based on activities per gram of substrate.

Activities	P. ostreatus[3] florida	P. sajor-caju[3] ATCC 32771	Hemicellulases: Enzeco Hemicellulase	Hemicellulase	SP249	Cellulases: Biocellulase	Cellulase A	Celluclast	Fungal Cellulase
Hemicellulases:									
Endo-1,4- -D xylanase[1]	10.20	5.81	610.00	34.1	299.70	605.70	1214.10	64.10	1260.70
Endo-1,4- -D mannanase[1]	1.26	2.24	0.00	20.0	0.00	0.00	30.0	7.10	0.90
ß-D Xylosidase[2]	28.00	8.00	6.00	9.00	0.00	0.00	0.00	0.00	0.00
Cellulases:									
Exo-1,4- -D glucanase[1] (filter paper)	0.89	1.25	134.20	103.00	42.50	345.20	155.10	123.20	184.40
Exo-1,4- -D glucanase[1] (Avicel cellulose)	0.21	0.08	2.80	1.60	2.30	9.90	7.30	8.00	0.70
Endo-1,4- -D glucanase[1]	2.04	2.54	148.10	79.10	8.10	403.00	268.60	19.70	332.00
ß-D glucosidase[1]	1.61	1.01	0.10	0.10	0.00	0.10	0.10	0.00	0.10
Protein (mg/g)	0.17	0.19	16.70	1.67	9.60	43.30	13.80	10.70	20.00

[1] µM/min/g/substrate
[2] mM/min/g/substrate
[3] Substrates were supplemented with 2% protein from wheat bran.

Table 9. Comparison of activities of Pleurotus fungi and commercial enzymes based on activities per gram protein.

Activities	P. ostreatus florida[3]	P. sajor-caju ATCC 32771[3]	Hemicellulases			Cellulases			
			Enzeco Hemicellulase	Hemicellulase	SP249	Biocellulase	Cellulase A	Celluclast	Fugal Cellulase
Hemicellulases:									
Endo-1,4- -D xylanase[1]	60.0	30.58	36.50	20.40	31.20	37.10	87.90	6.00	63.00
Endo-1,4- -D mannanase[1]	7.41	11.79	0.00	11.90	0.00	0.70	0.00	0.00	0.60
β-D Xylosidase[2]	165.00	42.00	0.36	5.39	0.00	0.00	0.00	0.00	0.00
Cellulases:									
Exo-1,4- -D glucanase[1] (filter paper)	5.23	6.58	8.00	61.70	4.40	7.90	11.20	11.50	9.20
Exo-1,4- -D glucanase[1] (Avicel cellulose)	1.23	0.42	0.20	0.90	0.20	0.20	0.50	0.70	0.00
Endo-1,4- -D glucanase[1]	12.00	13.37	8.80	48.40	0.80	9.30	19.40	1.80	16.60
β-D glucosidase[1]	9.47	5.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[1] μM/min/g protein
[2] mM/min/g protein
[3] Substrates were supplemented with 2% protein from wheat bran.

Table 10. Endo-1,4-β-D xylanase activities in enzyme preparates, μM/min/g product.

Product	Activity
Crude enzyme levels in the ground spent substrate	
P. sajor-caju	5.81
P. ostreatus florida	8.28
Enzyme concentrate obtained by air classification of ground spent substrate	
P sajor caju	
- light fraction	7.80
- heavy fraction	4.72
P. ostreatus florida	
- light fraction	12.11
- heavy fraction	5.07
Enzyme isolate obtained by water extraction of substrate and acetone precipitation of proteins	
P. sajor-caju	37.73
P. ostreatus florida	55.73

Appendix 1.

Experimental Design.

Wheat straw, var. Neepawa, agricultural lime and wheat bran from Robin Hood Multifoods, Ltd., in Saskatoon were used to formulate the substrate mixtures for experimental production of mushrooms.

The control substrates were formulated from wheat straw and agricultural lime only (Appendix 2). The experimental substrates were additionally supplemented with wheat bran to increase the protein content in substrate to 2%. Moisture contents of all substrate mixtures and controls were adjusted to 70%.

All substrates were composted for three days in a semi-aerobic conditions prior to sterilization in the autoclave at 121°C for 90 min.

The spawn of P. mushrooms was prepared at SRC, using rye grain adjusted to 50% moisture and sterilized at 121°C for 60 min prior to inoculation with the pure mushroom cultures. Spawn of three strains of P. sajor-caju and one strain of P. ostreatus var. florida was produced at 25°C in darkness.

Spawn was added to the sterile substrate mixtures and controls at 1.6, 2.4 and 3.2% of substrate wet weight and the inoculated substrates were bagged in clear plastic bags. Six bags were prepared for each treatment.

Inoculated bags were transferred to Allan Potash Mine of the Potash Corporation of Saskatchewan or to the SRC growthroom. An experimental growthroom was build in a closed corridor of the Allan Mine, about 1 km underground. A tent, approximately 22 x 8 x 7 ft., made of heavy duty plastic sheeting was suspended from the ceiling and walls of the mine corridors. The floor of the tent was lined with the same plastic. Three

fluorescent light fixtures, an exhaust fan and two humidifiers were enclosed within the tent structure and operated on timers.

A fully enclosed, insulated, walk-in growthroom was available in the SRC pilot plant. The growthroom has a floor area of 66 ft^2. and is equipped with an air conditioner, humidifiers, exhaust fan and fluorescent light fixtures. All electrical equipment was operated by timers.

The dark, vegetative phase of mushroom production was carried out for 12 days and was followed by a fruiting phase which required the control of the growth environment. Lights were turned on automatically for 8 hrs a day. Humidity in the growthroom areas was increased from 35-40% RH (air humidity) to about 70% RH by two to four humidifiers working continuously. To maintain low concentration of CO_2 in the growthrooms, an exhaust fan was operated for 10 min. during each hour.

First flush of mushrooms was harvested after 15-17 days, counting from the day of substrate inoculation, with subsequent flushes to follow at 5-7 day intervals.

Yield of the first flush of mushrooms harvested in the mine was 40% of dry substrate weight for control and 56% for substrate supplemented with protein. The corresponding rates obtained in the growthroom were 27 and 39%. Total mushroom yield was 65 and 95%, for control and protein supplemented substrates, resp., fruited in the mine and at 60 and 90%, resp., in the growthroom. Total production time in the mine was shorter than in the growthroom by 6 days.

Appendix 2

Composition of Substrate Mixtures for Production of Oyster Mushrooms (%)

Treatment	Inoculum % of substrate wet weight**	Substrate Components*		
		Straw var. Neepawa	Agricultural Lime	Wheat Bran
Control	1.6	96.0	4.0	-
1	1.6	86.5	4.0	9.5
2	2.4	86.5	4.0	9.5
3	3.2	86.5	4.0	9.5

* Misture of the substrate mixtures was adjusted to 70%
** Rye grain inoculum at 50% moisture.